

**GENETIC POLYMORPHISM IN THE RECEPTOR FOR IgA****Related Application**

This patent application is based on United States provisional application  
Serial No. 60/094,096 filed July 24, 1998, entitled "Genetic Polymorphism in the  
5 Receptor for IgA".

**Field of the Invention**

The present invention relates generally to compounds and methods for  
identifying polymorphism in a cellular receptor, and more particularly, to  
compounds and methods for identifying and typing single nucleotide  
10 polymorphisms that code for IgA receptors and applying these polymorphisms to  
delineation of disease susceptibility and severity.

**Background of the Invention**

Immunoglobulins are responsible for responding to antigenic biochemicals  
encountered by a host eukaryotic organism. Immunoglobulin binding by a host  
15 receptor is important in activating immunological responses to an antigen. As a  
result, polymorphic receptor variations between individual hosts is expected to  
have implications in the relative susceptibility of individuals to similar antigenic  
challenge.

Recent advances in our understanding of the molecular structure and  
20 diversity of immunoglobulin receptors have focused attention on opportunities for  
insights into their role in the pathogenesis of antibody-mediated acute and chronic  
inflammation.

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The IgG binding receptors have been extensively studied. These studies illustrate several aspects of receptor polymorphism that are important in understanding the utility of receptor genotyping individuals. Three distinct families of human receptors for the Fc domain of IgG (FcγRI, FcγRII, and FcγRIII) with different functional potential have been identified (1-3). Within these three FcγR families of related yet diverse molecules, distinct genes and alternative splice variants lead to a series of receptor isoforms that have striking differences in the extracellular, transmembrane and intracellular regions. In addition, most receptor isoforms have structurally defined allelic polymorphisms (4-9). The various FcγR isoforms have different functional capacities based on different functional domains and/or sequence motifs. Furthermore, allelic variants of FcγRIIa, FcγRIIIa and FcγRIIIb also have distinct functional capacities (Preliminary Results; 4,9-13). For example, the alleles of FcγRIIa, which differ at amino acid position 131, differ substantially in their capacity to ligate human IgG2 (9,10,12,13) while the F/V-176 alleles of FcγRIIIa differ in their capacity to ligate human IgG1 and IgG3 (4).

Human neutrophils constitutively express FcγRIIa and FcγRIIIb, both of which have functionally significant allelic variants. FcγRIIa has an ITAM signaling motif in the cytoplasmic domain (14), mediates signaling events that are dependent on both phosphorylation and dephosphorylation, and interacts with actin filaments suggesting direct involvement in phagocytosis (15). Although the functional capacity of FcγRIIIb has been more controversial, in a myeloid environment FcγRIIIb initiates a range of cell programs (9,15-17). Importantly,

the expression of FcγRIIIb is restricted to neutrophils (and eosinophils). FcγRI expression is inducible on neutrophils by IFNγ or IL-10 (18-20).

Human monocytes constitutively express FcγRIa and FcγRIIa; and with differentiation into monocyte-macrophages, they express FcγRIIIa and FcγRIIb.

5 FcγRIa and FcγRIIIa associate with FcεRI γ-chain which has an ITAM signaling motif, and both receptors can initiate degranulation, a respiratory burst and phagocytosis. FcγRIIb is distinct in having an inhibitory ITIM motif (21,22). Most carefully studied on B lymphocytes, FcγRIIb recruits phosphatases (SHP1 and SHIP) to its signaling complex and downregulates net signaling function (23-  
10 26).

In contrast to the Fcγ receptors, Fcα receptors are comprised of a single family of molecules most probably derived by alternative splicing from a single gene (27-32). The dominant protein product, FcαRIa (CD89), is a transmembrane protein which is heavily and variably glycosylated (33,34). Like FcγRIa and  
15 FcγRIIIa, FcαRIa lacks recognized signaling motifs in its cytoplasmic domain. It associates with FcεRI γ-chain which has an ITAM (35), but unlike FcγRIIIa, FcαRIa does not require the FcεRI γ-chain for expression. Similar to FcγRIa and FcγRIIIa, FcαRIa can initiate degranulation, a respiratory burst and phagocytosis at least in some experimental systems (36-38).

20 FcαRIa is constitutively expressed on human neutrophils and monocytes with about 6,000-7,000 copies per cell. Its expression is upregulated on activated neutrophils, including exudative neutrophils obtained from the gingival crevice and kidney glomeruli. Among cytokines, TNFα, IL-8 and GM-CSF increase

surface expression of Fc $\alpha$ RIa while IFN $\gamma$  and TGF $\beta$  downregulate it (39). Alleles of Fc $\alpha$ RIa are unexplored.

Immunoglobulin A (IgA) plays a prominent role in host defense at mucosal surfaces and in secretions such as tears, saliva and sweat where secretory IgA is the predominant immunoglobulin isotype. IgA appears to serve as a non-inflammatory regulator of the local immune response through the production of IL1ra and the absence of production of typical pro-inflammatory cytokines (40-44). These effects contrast with the well known anti-microbial defense functions of IgM and IgG which activate cell programs for inflammation and microbial destruction which may, in turn, injure surrounding host tissues. However, it is now recognized that receptors for IgA (Fc $\alpha$ R) and for IgG (Fc $\gamma$ R) depend upon tyrosine activation motifs for their function and that Fc $\alpha$ RI (CD89), Fc $\gamma$ RI (CD64) and Fc $\gamma$ RIII (CD16a) use the identical Fc $\epsilon$ RI  $\gamma$ -chain for signaling. Consequently, the critical question of how these  $\gamma$ -chain associated receptors generate such divergent cell programs comes sharply into focus. Indeed, in the gingival lesion of chronic periodontal disease (PD) where both IgAs and IgGs are abundant, the balance of pro-inflammatory and anti-inflammatory programs is critical. Identification of the pivotal anti-inflammatory signaling elements differentially engaged by Fc $\alpha$ RI provides a strategy for selective intervention and a target for therapeutic development. IgA through its prominent role in protecting surface tissues against invasion by pathogenic organisms is found throughout the respiratory, gastrointestinal and genitourinary tracts. The progression of diseases associated with these tracts is expected to relate to Fc $\alpha$ R function.

Periodontal disease serves as a model for other diseases involving IgA activity. These other diseases include systemic lupus erythematosus (SLE), systemic vasculitis, and IgA nephropathy.

Periodontal disease is a chronic, recurrent inflammatory condition initiated and sustained by subgingival plaque bacteria but defined by the host immune system's inflammatory response which results in destruction of structures supporting the teeth (45-50). While bacterial pathogens and their products can damage host supporting tissues, disease can also arise from an exuberant inflammatory immune response by the host. Normally, this response is self-limited and the organism is eliminated. Some individuals, however, seem more susceptible to persistent or recurrent gingival disease, and this may relate to inherent differences in their immune system response. Indeed, some mechanisms of PD may resemble other chronic inflammatory conditions even though PD's distinctive features depend on the unique characteristics of the gingiva, teeth, supporting structures and microorganisms that reside in the oral cavity.

Among the organisms associated with PD, *Porphyromonas gingivalis* has been strongly implicated in adult PD (51,52). *P. gingivalis*-derived proteases damage tissue directly, as well as indirectly through the induction of collagenase secretion and interruption of complement-mediated defenses (53-56). Despite subtle differences in the chemical structure and biological properties of its lipopolysaccharide (LPS) relative to that from enterobacteria, *P. gingivalis* LPS induces mononuclear phagocytes to release inflammatory cytokines including TNF $\alpha$ , IL-1, and IL-6 (57-60). Fimbriae and other surface components of *P.*

*gingivalis* also stimulate macrophages to produce IL-1 (61,62). Of the two isoforms of IL-1, IL-1 $\beta$  is prominent in inflamed gingiva (63).

5        Secretion of IL-1ra by monocytes is a critical anti-inflammatory counterbalance (64). IL-1ra is an analog of IL-1 that blocks the effect of IL-1 by competitively binding to IL-1 receptors without transducing an activation signal. IL-1ra production is induced in monocytes and macrophages by multiple stimuli, IFN $\gamma$ , IL-4, IL-6, IL-10 and bacterial LPS. IL-1ra production is also stimulated by IgG immune complexes through stimulation of Fc $\gamma$ RIIa (CD32) and Fc $\gamma$ RIIIa (CD16) (65-67), but the addition of LPS to Fc $\gamma$ R stimulated cells augments IL-1 $\beta$  and suppresses IL-1ra production (68).  
10        IgA immune complex stimulation of Fc $\alpha$ RI (CD89) leads to marked enhancement of IL-1ra production without IL-1 and TNF $\alpha$  (44). Since the ratio of IL-1ra:IL-1 has been implicated in adult periodontitis as well as several other inflammatory diseases including inflammatory bowel disease and systemic lupus erythematosus (69-71), the  
15        relative contributions of Fc $\gamma$ R and Fc $\alpha$ RI in stimulating local neutrophils and monocyte-macrophages is clearly of critical importance.

      Recent association studies have suggested that Fc $\gamma$  receptor polymorphisms (72-73) and that IL-1 $\alpha$ /IL-1 $\beta$  genotype (74) may be related to the risk and/or severity of periodontal disease. These insights underscore the  
20        importance of the characteristics of the host immune response in defining risk for chronic inflammatory diseases, such as periodontal disease. Since various microorganisms play a role in the etiology of periodontal disease, it is also reasonable to expect that the susceptibility to disease, the severity and response to

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treatment or resistance would be associated with the MHC polymorphisms which are intimately involved in antigen processing and presenting antigens to T-cells (75). For example, several HLA-A and B alleles are associated with periodontitis among various racial and ethnic groups (76). The strongest association reported was with HLA-B35 (RR = 6.03) in West Indies blacks. A similar association was observed between juvenile periodontitis and HLA-A33 and DR2 in African Americans under the age of 30 years (77). In a study of a Japanese family with early-onset periodontitis, high IgG titers were observed to *Porphyromonas gingivalis* and all subjects had HLA-DR52 and DQI in common (78-79). A study of renal transplant patients from Istanbul who received cyclosporine-A revealed that DRI was significantly increased in those who did not develop gingival overgrowth compared to those who did (80). However, in Italian transplant patients who received cyclosporin and nifedipine the frequency of HLA-A19 was increased in those with gingival overgrowth compared to those without but did not reach statistical significance (81).

The literature also includes observations that genetic polymorphisms in several FcγR expressed on myeloid cells may influence the recurrence and severity of PD (82,83). These observations raise the possibility that similar polymorphisms in FcαRI may play an important role in the development of PD. FcαRI has several different splice isoforms (27-31) and is extensively, yet variably, glycosylated (33-34).

It would thus be desirable to provide an assay for determining the extent to which FcαRI is also genetically polymorphic, and whether single nucleotide

polymorphisms (SNPs) lead to coding changes in both the extracellular and cytoplasmic domains. Characterization of the respective biologies of these SNPs is dependent on an understanding of the functional domains and molecular docking motifs of Fc $\alpha$ RI.

5           It would also be desirable to assay genetically determined and post-translationally modified variations in Fc $\alpha$ RI structure and to define the role of both extracellular and cytoplasmic domains essential for functions which may have significant impact on the progression of PD.

10           It would also be desirable to provide an assay to characterize the signaling elements associated with Fc $\alpha$ RI and their role in determining unique Fc $\alpha$ RI signaling events.

          It would also be desirable to identify novel SNPs affecting Fc $\alpha$ RI structure and function.

15           It would also be desirable to identify both pre- and post-translational variations and modifications of Fc $\alpha$ RI and their impact on Fc $\alpha$ RI function.

          It would also be desirable to establish the role of Fc $\alpha$ RI splice variants and of both Fc $\alpha$ RI and Fc $\alpha$ R polymorphisms in determining the ability of a host to fight certain bacterial and viral infections, the susceptibility to and/or severity of autoimmune diseases, as a prognosis indicator or to identify suitable vaccinations  
20           or treatments.

### **Summary of the Invention**

          The present invention is a system and method for correlating the ability of a cell to bind immunoglobulin (IgA) and cellular susceptibility to a disease by



identifying a Fc $\alpha$ RI genotype of a cell and quantifying IgA binding by the cell expressing the Fc $\alpha$ RI genotype. Thereafter, the IgA binding by the cell and IgA binding by a second cell expressing a second Fc $\alpha$ RI genotype is compared.

The present invention uses a single nucleotide polymorphism or combinations thereof within a Fc $\alpha$ RI genotype to identify individual susceptibility to a disease.

The methods of the present invention also extend to correlating the ability of a cell to bind IgA and cellular susceptibility to a disease through identifying a Fc $\alpha$ RI phenotype of a given cell and quantifying IgA binding by said cell. Thereafter, IgA binding by the cell is compared to a second cell having a second phenotype of Fc $\alpha$ RI. In particular, single nucleotide polymorphisms are responsible for genotypical and phenotypical differences in Fc $\alpha$ RI herein.

The present invention further includes a commercial packaging including reagents for identifying single nucleotide polymorphisms in the Fc $\alpha$ RI genotype or phenotype of an individual as a test to identify individual susceptibility to a disease. The reagents further include instructions for the use thereof.

#### **Brief Description of the Drawings**

Figure 1 - TGF $\beta$  causes a significant ( $p < 0.01$ ) decrease in Fc $\gamma$ RI specific phagocytosis in 18-hr cultured human monocytes. Phagocytosis by Fc $\gamma$ RII and Fc $\gamma$ RIII are unaffected by TGF $\beta$  but there is a modest increase in Fc $\gamma$ RIIIa expression. Data are reported relative to untreated cells. Phagocytosis normalized to expression is 0.5, 1.2 and 0.9 for Fc $\gamma$ RI, Fc $\gamma$ RII and Fc $\gamma$ RIII respectively.

Figure 2A - Receptor specific phagocytosis (E-22.2 F(ab)<sub>2</sub>) by transfected human wild type and mutant tail minus FcγRIa in P388D1 cells.

Figure 2B - Pre-incubation of transfected P388D1 cells with saturating levels of murine IgG2a and/or okadaic acid before addition of E-22.2 did not significantly alter FcγRIa phagocytosis. Binding of the E-22.2 is not affected by either pre-incubation.

Figure 3 - Differential affects of the calcium chelator BAPTA, the protein tyrosine kinase inhibitor genistein and the ser/thr phosphatase inhibitor okadaic acid on receptor specific phagocytosis mediated by transfected WT and MT FcγRI. Data are presented relative to control untreated cells.

Figure 4 - Cross-linking of transfected WT FcγRIa with receptor-specific mAb 22 induces production of both IL-1β and IL-6 while MT FcγRI only induces IL-1β. Data are presented as pg/ml above control unstimulated cells after 8 hours.

Figure 5 - Sequence of the cytoplasmic domain of FcαRI (CD89) showing potential kinase phosphorylation sites.

Figure 6 - Genomic organization of FcαRI (CD89) showing polymorphic sites.

Figure 7 - Blockade of binding of the anti-FcγRIII ligand binding site mAb 3G8 by increasing concentrations of human IgG. The level of mAb 3G8-FITC binding to FcγRIII after incubation with human IgG is shown.

Figure 8 - Serum creatinine values from patients with Wegener's Granulomatosis with renal disease.

**Detailed Description of the Invention**

The present invention provides methods for determining the allelic pattern for Fc $\alpha$ RI genes in human patients. The methods encompass the use of allele specific oligonucleotides as hybridization probes and/or as primers for DNA amplification, as well as the use of direct DNA sequencing. Identification of receptor alleles may also be achieved immunologically, by contacting mucosal cells that express Fc $\alpha$ RI receptors on their cell surface with antibodies that distinguish between different polymorphic forms of the receptor.

The present invention is based on the finding that allelic polymorphism in a gene encoding Fc $\alpha$ RI receptor isoform results in functionally distinct gene products. The expression of a polymorphic receptor in an individual may have important consequences for the physiological activity of the receptor and thus for the functioning of the cell types that carry the receptor. Because of the important immunological role of IgA in mucosal immunity, the present invention has utility as a diagnostic to identify high risk patients that warrant early and aggressive treatment. As a diagnostic for infectious disease, the present invention has utility in predicting neonatal sepsis and thereby guiding the use of therapeutic gamma globulin. As a diagnostic for autoimmune disease, the present invention has utility in diseases illustratively including systemic lupus erythematosus, systemic vasculitis, IgA nephropathy, rheumatoid arthritis, systemic sclerosis, dermatomyositis, Hashimoto's thyroiditis, inflammatory bowel disease and Sjogren's syndrome. For cancer, the present invention has utility as an immune

surveillance test for monitoring protective and nonprotective antibody levels in response to cancer therapies.

The present invention provides a method for identifying the Fc $\alpha$ RI allelic pattern in human patients which comprises testing mucosal tissue cells or DNA from individual patients for the presence of different Fc $\alpha$ RI allelic variants, using antibody based and/or nucleic based methods which are described in more detail below. The identification of receptor allelic patterns of the present invention further finds utility in identifying gene therapy as a means for providing more effective receptor alleles to a patient.

The present invention also encompasses the identification analysis of new allelic forms of Fc $\alpha$ RI genes, the analysis achieved using methods well known in the art, such as for example, DNA sequencing; single strand conformational polymorphism analysis (SSCP) (84); "HOT" cleavage (85); denaturing gradient gel electrophoresis (DVGE) (86) and combinations thereof. Once a new polymorphism has been identified, immunological and/or molecular biological tests are used to genotype patients for the presence or absence of the polymorphism. For example, monoclonal antibodies specific to the protein encoding for a newly identified allele are prepared by well known methods; these antibodies can be used for genotyping the patient populations as described above. Alternatively, allele specific oligonucleotides are designed for use as probes and/or as primers in hybridization or PCR-based detection methods, respectively.

The present invention characterizes the signaling elements associated with Fc $\alpha$ RI and their role in determining unique Fc $\alpha$ RI signaling events. Using the

yeast two-hybrid system with CD89 alpha chain cytoplasmic domain (CY) as "bait," novel molecular associations with the CY, the residues meeting these associations, and the potential dependence on serine/threonine phosphorylation are determined. Alternatively, the properties of CD89 alpha chain signaling alone, the regulation of  $\gamma$ -chain pairing with CD89 alpha chain, and the signaling elements engaged by CD89, as opposed to CD16 and CD64, are defined. A further method of characterizing the signal elements associated with Fc $\alpha$ RI includes using stably transfected constructions with specific deletions, truncations and point mutations of CD89. The effect of these constructs on associations, the role of these interactions in modulating signaling and initiation of cell programs is thereby determined.

The present invention also encompasses identification of novel single nucleotide polymorphisms (SNPs) affecting Fc $\alpha$ RI structure and function. SNPs are identified using direct cycle sequencing optimized for heterozygote detection, genomic DNA and cDNA obtained by RT-PCR from mRNA so as to provide a template to identify novel SNPs in normal donors, in PD, rheumatoid arthritis patients and in other patients suffering from diseases illustratively including connective tissue, autoimmune, bacterial and viral infection, and cancer. Through the establishment of statistically significant correlations between the different allelic forms of Fc $\alpha$ RI (and allelic patterns formed by combinations of different alleles) and various physiological and/or clinical manifestations of variable Fc $\alpha$ RI function, the role of naturally occurring point mutations in modulating signaling and initiation of cell programs is identified in homozygous genotype donors and

in stable transfectants. These correlations are utilized to provide the diagnostic utilities of the present invention. In practicing the present invention, preferably the correlations sought are those between particular Fc $\alpha$ RI allelic polymorphs and, for example: the risk for developing the aforementioned diseases; the effectiveness of antibodies protective against biologic- or tumor neo-antigens; and the risk of immune complex disease. In order to establish the impact of both pre- and post-translational variations and modifications of Fc $\alpha$ RI on Fc $\alpha$ RI function, the variation and expression of Fc $\alpha$ RI splice variants is determined in circulating and in crevicular fluid myeloid cells from patients suffering from an aforementioned disease in relation to a disease free control group. An RT-PCR method is used to identify translational variations and modifications of the receptor. Preferably, the Fc $\alpha$ RI "b" form is investigated for correlations through the use of different TM/CY. Gel electrophoresis of immunoprecipitated Fc $\alpha$ RI from cell lines and *ex vivo* myeloid cells is used to identify different receptor molecular weights, as previously done with Fc $\gamma$ RIIIa (17). Gel electrophoresis serves to characterize the glycosylation state in relationship to the ligand binding ability of the receptor polymorph. Translational variations and modifications of receptor glycosylation in patients suffering from an IgA related immune response disease can be correlated with SNPs of the receptor, as well as with the glycosylation status of both IgA and IgG.

Statistical methods, illustratively including a 2x3 chi-square test is used to determine allele frequencies in disease and control groups. In this manner, it is possible to obtain statistically significant correlations between a given disease and

Fc $\alpha$ RI alleles and thereby a diagnostic as to disease susceptibility and clinical outcome.

In practicing the present invention, the Fc $\alpha$ RI allelic pattern in an individual patient is determined by either: 1) immunological detection of one or more allelic forms of Fc $\alpha$ RI polypeptides present on the surface of appropriate immune cells ("phenotypic characterization"); or 2) molecular detection of the DNA or RNA encoding one or more Fc $\alpha$ RI allelic forms using nucleic acid probes, with or without nucleic acid amplification or sequencing ("genotypic characterization").

In one method, myeloid cells, mucosal cells, or those of any tissue expressing CD89 or subsets thereof are isolated from a patient to be tested using methods that are well known in the art, such as, for example, gradient centrifugation and/or immunoadsorption. Antibodies that are capable of distinguishing between different allelic forms of Fc $\alpha$ RI are then applied to the isolated cells to determine the presence and relative amount of each allelic form. The antibodies may be polyclonal or monoclonal, preferably monoclonal. Measurement of specific antibody binding to cells may be accomplished by any known method, including without limitation quantitative flow cytometry, or enzyme-linked or fluorescence-linked immunoassay. The presence or absence of a particular allele, as well as the allelic pattern (i.e. homozygosity vs. heterozygosity) is determined by comparing the values obtained from the patient with norms established from populations of patients of known genotypes.

In an alternate embodiment, DNA is obtained from a patient, and the presence of DNA sequences corresponding to particular Fc $\alpha$  receptor alleles is determined. The DNA may be obtained from any cell source or body fluid. Non-limiting examples of cell sources available in clinical practice include blood cells, buccal cells, cervicovaginal cells, epithelial cells from urine, fetal cells, or any cells present in tissue obtained by biopsy. Body fluids include blood, urine, cerebrospinal fluid, and tissue exudates at the site of infection or inflammation. DNA is extracted from the cell source or body fluid using any of the numerous methods that are standard in the art. It is understood that the particular method used to extract DNA will depend on the nature of the source.

Once extracted, the DNA may be employed in the present invention without further manipulation. Alternatively, the DNA region corresponding to all or part of the Fc $\alpha$ RI may be amplified by PCR or other amplification methods known in the art. In this case, the amplified regions are specified by the choice of particular flanking sequences for use as primers. Amplification at this step provides the advantage of increasing the concentration of Fc $\alpha$ RI DNA sequences. The length of DNA sequence that can be amplified ranges from 80 bp to up to 30 kbp. Preferably, primers are used that define a relatively short segment containing sequences that differ between different allelic forms of the receptor.

The presence of Fc $\alpha$  receptor allele-specific DNA sequences may be determined by any known method, including without limitation direct DNA sequencing, hybridization with allele-specific oligonucleotides, and single-stranded conformational polymorphism (SSCP). Direct sequencing may be



accomplished by chemical sequencing, using the Maxam-Gilbert method (87), or by enzymatic sequencing, using the Sanger method (88). In the latter case, specific oligonucleotides are synthesized using standard methods and used as primers for the dideoxynucleotide sequencing reaction.

5            Preferably, DNA from a patient is subjected to amplification by polymerase chain reaction (PCR) using specific amplification primers, followed by hybridization with allele-specific oligonucleotides. Alternatively, SSCP analysis of the amplified DNA regions may be used to determine the allelic pattern. Most preferably, allele-specific PCR is used, in which allele-specific  
10           oligonucleotides are used as primers and the presence or absence of an amplification product indicates the presence or absence of a particular allele.

            In an alternate embodiment, cells expressing Fc $\alpha$ RI are isolated by immunoadsorption, and RNA is isolated from the immunopurified cells using well-known methods such as guanidium thiocyanate-phenol-chloroform extraction  
15           (89). The isolated RNA is then subjected to coupled reverse transcription and amplification by polymerase chain reaction (RT-PCR), using allele-specific oligonucleotide primers. Conditions for primer annealing are chosen to ensure specific reverse transcription and amplification; thus, the appearance of an amplification product is diagnostic of the presence of the allele specified by the  
20           particular primer employed. In another embodiment, RNA encoding Fc $\alpha$ RI is reverse-transcribed and amplified in an allele-independent manner, after which the amplified Fc $\gamma$  receptor-encoding cDNA is identifiable by hybridization to allele-specific oligonucleotides or by direct DNA sequencing.

The term "allelic form" is intended to mean an alternative version of a gene encoding the same functional protein but containing differences in nucleotide sequence relative to another version of the same gene.

5 The term "allelic polymorphism" or "allelic variant" is intended to mean a variation in the nucleotide sequence within a gene, wherein different individuals in a general population express different variants of the gene.

The term "predetermined polymorphic DNA sequence" is intended to mean a known allelic variant.

10 The term "allelic pattern" is intended to mean the identity of each of the two copies of a particular gene in a patient i.e., homozygosity or heterozygosity.

The term "allelic pattern" is used herein interchangeably with "genotype."

The term "genotyping" as used herein as being the process of determining the allelic patterns of a human individual.

15 The term "point mutation" as used herein is intended to mean a mutation involving a single nucleotide.

The term "silent mutation" as used herein is intended to mean a change of a nucleotide within a gene sequence that does not result in change in the coded amino acid sequence.

20 The term "missense mutation" as used herein is intended to mean a change of a nucleotide within a gene sequence that results in a change in the meaning of a codon, thereby changing the coded amino acid.

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The term "frame shift mutation" as used herein is intended to mean a mutation involving the insertion or deletion of a single nucleotide that results in the remaining downstream sequence being transcribed or translated out of phase.

The term "amplification" in regard to DNA is intended to mean the use of  
5 polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences (90).

The term "chemical sequencing" in regard to DNA is intended to mean a method in which DNA is randomly cleaved using individual base specific reactions, illustratively including Maxam-Gilbert sequencing.

10 The term "enzymatic sequencing" in regard to DNA is intended to mean a method in which single stranded DNA is copied and randomly terminated using DNA polymerase, illustratively including the method of Sanger (88).

The term "effective Fc $\alpha$ RI mediated immune response" is intended to mean immune response that results in the lessening of at least one symptom of a  
15 disease to which the host immune response is directed.

While the description of the present invention that follows is largely directed to periodontal disease, it is appreciated that the receptor mediated immune response is similar in that of other human diseases of tissues protected by neutrophils and monocytes. The diseases illustratively including systemic lupus  
20 erythematosus, systemic vasculitis, IgA nephropathy, rheumatoid arthritis, systemic sclerosis, dermatomyositis, Hashimoto's thyroiditis, inflammatory bowel disease and Sjogren's syndrome.

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The insights into the structure and critical function of FcRs (91-93) and into the genetics of FcγRs in periodontal disease (82,83), coupled with an appreciation of the capacity of phagocytes to release cytokines and other inflammatory mediators, suggest that FcR-mediated triggering of neutrophils and Mφ is a central convergence point in PD. From this perspective, FcγR initiate synthesis and secretion of pro-inflammatory cytokines, release of degradative enzymes and release of reactive oxygen intermediates capable of tissue damage (94-96). As counterpoint, IgA immune complex stimulation of FcαRI (CD89) leads to marked enhancement of IL-1ra production without IL-1 and TNFα (44).

**Role of the cytoplasmic domain of the ligand binding α-chain in modulating receptor function.** Co-transfection and co-immunoprecipitation studies indicate that FcαRI (CD89), FcγRIa (CD64) and FcγRIIIa (CD16a) associate with FcεRI γ-chain (2,86). FcεRI γ-chain contains an immunoreceptor tyrosine activation motif (ITAM), is actively tyrosine- and serine/threonine-phosphorylated, and plays a critical role in function (2,14,98,99). Extrapolating from receptor chimera data and from experiments with point mutagenesis of receptor associated ITAMs, many investigators have concluded that FcεRI γ-chain is both necessary and sufficient for signal transduction (3,100). Indeed, within the field of Fc receptors, there is the common presumption that FcεRI γ-chain is the exclusive signaling pathway for those receptors which associate with it.

However, other evidence suggests that FcεRI γ-chain is not the sole actor in FcεRI γ-chain-associated Fc receptors. In 1993, Hogarth and colleagues

demonstrated that the cytoplasmic domain (CY) of murine FcγRI ligand binding chain (CD64; α-chain) is actively phosphorylated after treatment of J774 cells with phorbol myristate acetate (101). Transfection studies suggest that FcγRI, expressed without γ-chain, appears to be able to mediate some functions like endocytosis (102-104). The present invention demonstrates that the α-chain CY, despite the absence of recognized signaling motifs, participates in initiating cell programs. The FcγRI α-chain CY does interact with actin binding protein (105) and a naturally occurring FcγRI α-chain tail-minus mutant in the NOD mouse has some functional differences (106). While human blood monocytes incubated for 18 hrs with rIFNα show a modest increase in both FcγRI-specific and FcγRIII-specific phagocytosis, TGFβ causes a significant decrease in FcγRI mediated phagocytosis yet no effect on FcγRIII (Figure 1).

**Contribution of α-chain CY to FcγRI function.** P388D1 cells stably transfected with human wild-type (WT) and human CY-minus (MT) FcγRI are studied. Both cells lines express comparable levels of receptor. Like the truncated receptor in the NOD mouse, albeit more subtly, the MT in P388D1 endocytoses more slowly than WT. The MT phagocytoses less (Fig 2A), an effect which is independent of engagement of the ligand binding site (Fig 2B); it shows a greater sensitivity to chelation of  $[Ca^{2+}]_i$  and to the PTK inhibitor genistein and is resistant to the effects of okadaic acid (Fig 3), suggesting a differential engagement of signaling elements compared to WT. Furthermore, while both WT and MT synthesize IL1β, MT is markedly deficient in IL6 production at 8 hrs (Fig 4).

A model is suggested by the recognition that cytoplasmic domains of different associated chains can interact and modulate function (14,107,108). For example, in the FcεRI complex which is comprised of one α, one β and one γ homodimer, both the src family tyrosine kinase Lyn and the PKC isoform delta are constitutively associated with β which in turn facilitates tyrosine phosphorylation and threonine phosphorylation on γ, respectively (98,109,110). Although the FcγRIa complex does not have a corresponding β chain, the CY of the α-chain is involved in the interaction between the α-chain and may serve to recruit (or perhaps impede) signaling element(s); the okadaic acid data suggests that this element(s) may at least involve ser/thr phosphorylation. Of course, since the primary sequence of the α-chain CY domains for FcγRI, FcγRIII, and FcαRIa are different (Table 1), the contributions of each CY domain may provide the basis for the unique receptor function.

Inspection of the FcγRIII CY domain suggests several potential ser/thr phosphorylation sites (PKC motif: SSTRD and casein kinase 2: SSTRDW) and the present invention shows data parallel to that on FcγRIa, demonstrating that the CY domain is actively phosphorylated *in vitro*. The observation by Geisler and colleagues (111) that truncation FcγRIIIa CY alters the ability of the receptor to induce calcium flux further supports a role for the CY, and suggesting that Eibl's data (44) showing FcαRI stimulates primarily IL1ra and not TNFα production can be explained by a unique CY mechanism of the present invention.

To explore the mechanism and role of the CY by identifying intracellular interactions between proteins, the yeast two-hybrid system is used in the present

invention with the GAL4 transcriptional activator, with the CY domain as "bait" cloned in-frame into the pGBT9 vector containing the DNA binding domain of the GAL4 transcriptional activator and with three distinct cDNA libraries cloned into pACT with the GAL4 activation domain. As human cDNA libraries, an EBV-  
5 transformed PBMC library, a PHA-stimulated PBMC library and an unstimulated PBMC library have been used. Recognizing that achieving the native conformation of the CY domain is the goal in the two hybrid system, three different binding domain constructs: (1) binding domain - CY, (2) binding domain - 6 glycines - CY, and (3) binding domain- 6gly-CY-6gly-CY have been  
10 constructed. With this approach, a novel member of the band 4.1 family which associates with FcγRI CY but not with FcγRIII CY, CR1 CY and several other control baits has been identified.

Examination of FcαRIa CY sequence reveals that it is quite distinct from the other FcεRI γ-chain associated receptors (Table I) and that it contains several  
15 potential serine phosphorylation sites (Figure 5). In the two hybrid system, the present invention uses the native CY (with or without the 6 glycine linker), the CY with S to D/E to "mimic" serine phosphorylation, and then a variety of truncations and point mutations to establish the role of docking regions and sites with phosphorylation motifs.

20 Based on the precedent of biologically important SNPs in the coding regions of FcγRIIA, FcγRIIA and FcγRIIB, on the recognition of the influence of the FcγRIIA and FcγRIIB polymorphisms on the risk for PD, and on the knowledge that PD lesions are rich in both IgG and IgA, the present invention

identifies novel SNPs in Fc $\alpha$ RIa. Morton and colleagues have reported a silent SNP in codon 87 (AGA $\rightarrow$  AGG, remains Arg), an Asp $\rightarrow$  Asn in codon 92 (GAC $\rightarrow$  AAC) of extracellular domain 1 (EC1) and a Phe $\rightarrow$  Leu at codon 132 (TTT $\rightarrow$  CTT) of EC2 (112). Using direct cycle sequencing of cDNA derived from mRNA, Fc $\alpha$ RIa of 39 normals and 31 patients with rheumatoid arthritis (RA) were examined. However, of much greater interest are two novel polymorphisms of the present invention in the CY domain,-- a Pro $\rightarrow$ Leu at residue 245 and a Ser $\rightarrow$ Gly at residue 248 (Figure 6). The Pro $\rightarrow$ Leu is very uncommon with a gene frequency in our population suggesting that it will be classified as a mutation. The Ser $\rightarrow$ Gly polymorphism has gene frequencies of 0.874 and 0.126 respectively with no difference between normals and RA patients. Inspection of the full sequence of the Fc $\alpha$ RIa CY indicates that both the Pro and the Ser are part of a putative casein kinase I phosphorylation site (Figure 6). Thus, the present invention shows that there are FcR polymorphisms which directly affect signal transduction rather than ligand binding as the mechanism for altered function.

Gamma ( $\gamma$ ) chain plays a critical role in the function of Fc $\gamma$ RIa, Fc $\gamma$ RIIIa and Fc $\alpha$ RI (2). Through physical association in the plasma membrane with the ligand-binding alpha chain of these receptors,  $\gamma$ -chain provides a necessary signaling element(s) including an ITAM (immunoregulatory tyrosine-based activation motif) (14). The role of the phosphorylated tyrosine residues (pY) is to increase the binding affinity for SH2-containing molecules, but sequence analysis of the  $\gamma$ -chain cytoplasmic domain reveals multiple serine and threonine residues in/near the ITAM. Since the thr residues are in the ITAM [ $\gamma$ -chain CY:



RLKIQVRKAAITSYEKSDGVYTGLSTRNQETYETTLKHEKPPQ] which predicts direct contact with SH2-containing molecules attempting to "dock," one might imagine these residues as important modulators of this process.

Fc $\alpha$ RIa is known to be differentially glycosylated in different cell types (33,34) and it has been shown that an altered glycosylation pattern of receptor in certain diseases such as IgA nephropathy. Work with Fc $\gamma$ RIIIa expressed on NK cells and on monocytes-macrophages provides a framework which suggests that these glycosylation differences in Fc $\alpha$ RIa are important. Fc $\gamma$ RIIIa expressed on NK cells and on monocyte-macrophages is also differentially glycosylated despite identical protein cores (Table 3) and these different glycoforms have different affinities for ligand (Figure 15). The macrophage form has a lower apparent Mr, is lower in sialic acid content, and has a lower affinity for IgG (113). The broad range of apparent Mr which is greater than can be explained by known splice isoforms of Fc $\alpha$ RI is consistent with disease-associated changes in glycosylation altering receptor function.

Several lines of evidence suggest that Fc receptor polymorphisms are important in the development of disease phenotype in chronic inflammatory diseases. The present invention shows that alleles for both Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa are altered in their representation in SLE with low binding alleles associated with the SLE phenotype and high binding alleles (176V) protecting against glomerulonephritis (Table 2) (4,114). It is also shown that alleles of Fc $\gamma$ RIIIB predict the severity of glomerulonephritis in ANCA-positive vasculitis such as Wegener's Granulomatosis (Figure 8). The data presented herein shows that the

novel Ser-Gly in the CK1 site of the Fc $\alpha$ RI CY domain is markedly enriched in chronic inflammatory disease (SLE) (Table 3).

Fc receptor polymorphisms are also important in host defense against infectious disease (115-117). Fc $\gamma$ RIIa, Fc $\gamma$ RIIIa, Fc $\gamma$ RIIIb and Fc $\alpha$ RIa are  
5 expressed on neutrophils and mononuclear phagocytes, and myeloid cells essential for host defense. Since Fc $\gamma$ RIIa-R131 binds human IgG2 poorly and IgG2 is a component of the host immune response to encapsulated bacteria, individuals homozygous for Fc $\gamma$ RIIa-R131 may be at greater risk for bacterial infection. Similarly, individuals homozygous for the low activity Fc $\gamma$ RIIIb-NA2 may be at  
10 greater risk for infection (118). Two initial reports (82,83) of associations of Fc $\gamma$ RIIa and Fc $\gamma$ RIIIb polymorphisms with PD phenotype (susceptibility, severity, recurrence) suggest that Fc $\gamma$ R-related function may be important in the pathogenesis of PD. However, while these reports in PD are suggestive, neither has considered the role of the novel Fc $\alpha$ RIa polymorphisms described above.  
15 Taken together, this repertoire of genetic variants provides a tool to anticipate phenotype, especially with regard to severity of disease.

The present invention characterizes the signaling elements associated with Fc $\alpha$ RI and their role in determining unique Fc $\alpha$ RI signaling events. Current evidence has been interpreted as demonstrating that the  $\gamma$ -chain ITAM is both  
20 necessary and sufficient for the function of the Fc $\epsilon$ RI $\gamma$ -chain associated Fc $\gamma$  receptors (CD16 and CD64). However, the present invention shows that the cytoplasmic domains of the ligand binding  $\alpha$ -chains in these  $\gamma$ -chain receptor complexes are functionally important and provide the basis for diversity in

signaling among these FcεRIγ-chain associated receptors. The Fc receptor for IgA also forms a receptor complex with the γ-chain, and there is clear precedent that this Fcα receptor complex is functionally distinct from the Fcγ receptor complexes. Accordingly, the present invention shows that the cytoplasmic domain (CY) of the ligand binding α-chain of CD89 can modulate the functional responses of the receptor complex and/or possibly elicit functional responses independently of the γ-chain.

**Functional impact of FcεRIγ-chain pairing with FcαRI α-chain.**

FcαRI has some homology with FcγR encoded on human chromosome 1 and with the KIR family of receptors found proximate to FcαRI on human chromosome 19 (119). Members of the KIR protein family are not known to associate with γ-chain. To determine the functional properties of FcαRI expressed as a protein associated or not associated with FcεRIγ-chain. For these studies, transfection uses wild type FcαRI and a mutant form of FcαRI that does not associate with γ-chain into an appropriate cell line. Previous studies in a double transfection system using the γ-chain lacking B cell line IIA1.6 has shown that Arg209 in the TM domain of FcαRI is required for association with the γ-chain (35). Confirmation of association or non-association of γ-chain in FcαRI immunoprecipitates from digitonin lysates of transfected cells utilizes a polyclonal anti-γ-chain Ab.

The choice of cell line for these studies is crucial because the cell line must be capable of performing appropriate functions when stimulated, and possess the necessary intracellular constituents to interact with FcαRI. For example,

engagement of Fc $\alpha$ RI is known to activate the src-family tyrosine kinase lyn (120,121), to activate the tyrosine kinase syk (120,121), to induce secretion of IL-1ra and to inhibit LPS induced secretion of IL- $\beta$  (and TNF $\alpha$ )(44). Suitable cell lines illustratively include: the murine monocyte-macrophage-like cell lines J774, 5 P388D1 and myeloid cells capable of eliciting appropriate functional responses. Suitable cell lines such as these are transfectable with plasmids and can express transfected human gene products (2,122,123); endogenously express the  $\gamma$ -chain (2). P388D1 has been used extensively to analyze the structural requirements for initiation of functional responses by human Fc $\gamma$  receptors (especially Fc $\gamma$ RIa and 10 Fc $\gamma$ RIIa) and is the preferred choice of host cell for Fc $\alpha$ RI transfections.

Activation of both early signaling events and cell programs are examined in stably transfected lines expressing the wild type and mutant non- $\gamma$ -chain associated forms of Fc $\alpha$ RI. Early signals include the phosphorylation of the src-family kinase lyn and of syk, association of these kinases with the receptor, and 15 activation of kinase activity. Based on the finding that another Fc $\epsilon$ RI $\gamma$ -chain associated receptor, Fc $\gamma$ RIa, can active both the conventional and the G-protein dependent for of PI $_3$  kinase (124), the present invention examines the capacity for CD89 to activate similar or dissimilar pathway(s). To this end, examinations occur of CD89 receptor specific [Ca $^{2+}$ ] $_i$  fluxes and both endocytosis and 20 phagocytosis at low and high levels of receptor cross-linking.

It is appreciated that a form of Fc $\alpha$ RI that does not associate with  $\gamma$ -chain can be produced by using a chimeric receptor. To maintain as much of the Fc $\alpha$ RI structure as possible, a variant of Fc $\alpha$ RI is constructed from which the TM domain

is removed and replaced it with CD8. CD8 does not associate with  $\gamma$ -chain. In analogy to the TCR and BCR (125), in some mutants, the magnitude of cross-linking of the receptor complex alters the functional consequences of receptor engagement. In particular, a higher level of receptor cross-linking (induced by  
5 IgA + F(ab')<sub>2</sub> anti-IgA and/or anti-receptor mAb + F(ab')<sub>2</sub> GAM) can result in a distinct pattern of cell activation. Using a biotin-avidin cross-linking system, one can titrate the stoichiometry of receptor aggregations (126).

The ability of different constructs of Fc $\alpha$ RI (carrying specific deletions, truncations, and point mutations) to initiate leukocyte cell functions is initially  
10 evaluated in cell lines that have been stably transfected with plasmids that express different forms of Fc $\alpha$ RI in association with  $\gamma$ -chain. Mutations in the CY domain of Fc $\alpha$ RI are guided by for instance functionally important regions and/or motifs in Fc $\alpha$ RI for novel associated molecules identified in the two hybrid system. Comparisons are made between cells that stably express on their  
15 membrane equivalent levels of native Fc $\alpha$ RI (full-length isoform), an Fc $\alpha$ RI deletion mutants lacking the CY domain and Fc $\alpha$ RI mutants having truncations or point mutations within the CY domain. Controls include cells not transfected with the CD89-expressing plasmid, or with the parent plasmid that does not contain CD89 DNA.

20 An aspect of present invention is the quantification of polymorphic differences in the induction of both rapid signals, -- endocytosis, receptor specific [Ca<sup>2+</sup>]<sub>i</sub> fluxes, activation of tyrosine kinase activity [lyn and syk], -- and of Fc $\alpha$ RI-specific effector functions. Receptor specific phagocytosis is evaluated using

latex microspheres or erythrocytes opsonized with human IgA or anti-Fc $\alpha$ RI mAb Fab/F(ab')<sub>2</sub> fragments to avoid co-ligation of endogenous Fc $\gamma$  receptors. Receptor specific stimulation of oxidative metabolism (superoxide production) are quantitated using the same probes as will cytokine production, especially IL-1ra, IL-1, IL-6, TNF $\alpha$ , which are relevant to macrophage function as mediators of inflammatory responses.

Engagement of Fc $\gamma$ R is a potent stimulus for the secretion of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and TNF $\alpha$  (2) and engagement of Fc $\alpha$ RI is known to attenuate release of these cytokines in response to LPS (44). Thus, Fc $\alpha$ RI expressing transfectants of the present invention are stimulated with surface bound IgG alone or in combination with surface absorbed IgA. Controls include stimulation with LPS in the presence and absence of surface absorbed IgA. Alternatively, mAb is used to engage distinct receptors on the cell surface. For example, mAb 2.4G2 is used to engage murine Fc $\gamma$ RII/Fc $\gamma$ RIII and/or murine IgG2a to engage murine Fc $\gamma$ RIa with and without anti-Fc $\alpha$ RI mAb Fab/F(ab')<sub>2</sub> fragments. Heterotypic and homotypic cross-linking is provided by either surface absorbed or solution phase F(ab')<sub>2</sub> goat anti-mouse IgG (GAM) or a combination of GAM and anti-human IgA.

#### **Functional significance of SNPs in the coding regions of Fc $\alpha$ RI.**

Peripheral blood neutrophils and monocytes, both of which express CD89, are obtained from genotyped donors and studied *ex vivo*. Peripheral blood monocytes are also be differentiated *in vitro* into macrophages resembling those found in tissues and having greater abilities to perform phagocytic functions.

The approach to the functional consequences of the CD89 SNPs varies with the location of the polymorphism in the protein. For polymorphisms in the extracellular region, differences in quantitative binding of IgA ligand to mononuclear and polymorphonuclear cells from genotyped homozygous donors  
5 are studied. Binding of both serum IgA and secretory IgA is determined by flow cytometry (127, 128).

The functional significance of the polymorphisms in the CY domain is determined by examination of Fc $\alpha$ RI induced functions. For example, the polymorphisms at positions 245 and 248 alter a casein kinase I phosphorylation  
10 site (Figure 7). For these studies, genotyped homozygous donors and mAb cross-linking are utilized to rule out any impact of other extracellular polymorphisms. Using neutrophils and monocytes from genotyped homozygous disease free donors, a comparison is made between activation of tyrosine kinase activity (lyn and syk) and the induction of cell programs such as receptor specific phagocytosis,  
15 oxidative burst, and cytokine production. The release of collagenase from monocytes as an enzyme relevant to periodontal tissue destruction is quantified by ELISA (129). In donors that are identical in the extracellular domains but homozygous for different alleles in the CY domain, IgA is also used as a stimulus, similar to the Fc $\gamma$ RIa system for ligand induced stimulation to differ quantitatively  
20 from that induced by cross-linked mAb (130).

After definition of functional differences in the donor population, stable transfectants of CD89 of various genotypes are used to more carefully characterize the full impact of each polymorphic residue on the functional properties of the

receptors. The choice of cell type is dictated in part by the functional consequences of the polymorphism.

**Identification of both pre- and post-translational variations and modifications of Fc $\alpha$ RI and their impact on Fc $\alpha$ RI function.** Fc $\alpha$ RI has

5 several splice variants and some investigators have hypothesized that these variants may have different functional capacities (29,30,32,112,113). No systematic evaluation of these splice variants in disease states has been previously undertaken to establish their potential relevance. Similarly, Fc $\alpha$ RI is known to be differentially glycosylated in different cell types and in different disease states  
10 (128,132). Using the glycoforms of Fc $\gamma$ RIIIa as a model (113), the present invention shows that these alterations of glycosylation of Fc $\alpha$ RI have different functional properties.

In order to more fully demonstrate the advantages arising from the present invention, the following examples are set forth. It is to be understood that the  
15 following is illustrative only and not intended as a limitation on the scope of the invention.

**Example 1 - Isolation of DNA/RNA and cDNA preparation:** Genomic DNA is extracted from leukocytes (in EDTA anti-coagulated whole blood) using with the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). RNA  
20 is isolated from whole blood leukocytes (or Ficoll-Hypaque separated leukocyte fractions) using Triazol RNA isolation solution (Gibco BRL, Gaithersburg, MD). cDNA is synthesized from 5-10 $\mu$ g RNA with the SuperScript Preamplification System (Gibco).



**Example 2 - cDNA sequencing:** To facilitate heterozygote detection, a dye primer strategy is used for fluorescence-based automated cycle sequencing of PCR products on an ABI 377. All sequencing primers are designed with an 18bp M13 sequence tag (ABI PRISM™ Dye Primer Cycle Sequencing -21M13 FS and M13REV FS Ready Reaction Kits (ABI, Foster City, CA)). The PCR products are purified with the QIAquick Gel Extraction Kit (Chatsworth, CA). The BigDye terminator sequencing reaction chemistry (ABI) is also used to detect heterozygosity in multiple Fc receptors genes including CD89. This chemistry has the advantage of allowing longer sequence reads and does not require the incorporation of additional M13-based sequence in the primers. It is appreciated that all potentially polymorphic residues must ultimately be confirmed by sequencing in both the forward and reverse directions using the dye-primer based strategy.

**Example 3 - Determination of Fcα receptor alleles (12-14):** Allele-specific PCR assays are used to genotype donors for the FcαRI alleles illustratively including: FcαRIa (EC1) - 87R/87R, FcαRIA (EC1) - 92D/92N, FcαRIa (EC2) - 132F/132L, FαRI (CY) - 245P/245L and FcαRI (CY) - 248S/248G alleles. Since there is a finite error rate in any genotyping assay, each assay is corroborated by direct dye-primer based cycle sequencing of at least 40 homozygous donors and an equal number of heterozygous donors. In addition, each assay includes blinded but known genotyped controls to verify the fidelity of the assay. Ambiguities in the assay are resolved by first repeating the allele-

specific PCR reaction and then by direct dye-primer based cycle sequencing of genomic DNA samples.

**Example 4 - Amplification of Fc $\alpha$ RI cDNA:** Two sets of overlapping primers incorporating either the M13 universal or reverse primer sequences at the appropriate 5' ends are used to amplify the entire coding region of the Fc $\alpha$ RI cDNA. PCR is performed in a GeneAmp 9600 PCR system with 2.5U of Taq DNA polymerase (Gibco BRL) using the following cycling conditions: 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec and extension at 72°C for one min, followed by a final extension at 72°C for ten min. The PCR products are gel purified with the QIAquick GEL Extraction Kit (Qiagen). The first set of primers including the underlined M13 sequence amplify from nt 34 in S1 to nt 563 in EC2 (forward primer: 5'-TGT AAA ACG ACG GCC AGT AGC ACG ATG GAC CCC AAA CAG-3' (SEQ ID NO. 1); reverse primer: 5'-CAG GAA ACA GCT ATG ACC GGT GTT CCC CAC TTT GGT GC-3' (SEQ ID NO. 2). The second set of primers amplify from nt 458 in EC1 to nt 934 in the 3'-untranslated region (forward primer: 5'-TGT AAA ACG ACG GCC AGT AGA ATA TTT CCC TCA CGT GC-3' (SEQ ID NO. 3); reverse primer: 5'-CAG GAA ACA GCT ATG ACC CTG GCT CCT CTC TGC CTT CAC-3' (SEQ ID NO. 4)).

**Example 5 - Yeast 2-Hybrid System:** The yeast two-hybrid system based on the Gal4 transcriptional activator will use our cloned 41 amino acid CD89 cytoplasmic domain in the pGBT9 vector containing the DNA binding domain of the GAL4 transcriptional activator (BD-CD89). Screening the BD-CD89 is

performed against a cDNA expression library isolated from total unstimulated human PBL, a cDNA library from EBV infected pooled human PBL and a cDNA library from PHA-stimulated PBL all of which are cloned into the pACT vector with the Gal4 activation domain. A U937 myeloid cDNA library in pACT is also constructed for this screening purpose. Using strain HF7c yeast His/Trp/Leu auxotrophs, double transformants (BD-CD89 + AD construct) are screened on Trp<sup>-</sup>, Leu<sup>-</sup>, His<sup>-</sup> dropout plates containing 10 mM 3-amino-triazole to select for positive interactions between the CD89 cytoplasmic domain and the fusion protein encoded by AD-cDNA. Growth positive colonies are screened secondarily for LacZ activity, the second reporter gene under the control of the Gal4 promoter. The plasmids containing the AD-cDNA construct is recovered from growth (+), LacZ (+) colonies by transforming into E. coli HB101, harvested, sequenced, and compared to information currently in GenBank. Likely candidates, identified by multiple isolates and by putative biology, are directly rescreened (BD-CD89 + AD-candidate cDNA), and, if positive, are then tested for protein-protein interactions in co-immunoprecipitations from whole cell lysates and in GST-fusion protein (Pharmacia) affinity matrix/whole cell lysate interactions. Positive candidates in the two hybrid screen which are also positive for *bona fide* protein-protein interactions are deemed to be true associations. An unstimulated PBL library in the DupLEX-A<sup>TM</sup> system (Origene) also successfully screened with a BD-FcγRIIIa cytoplasmic domain construct. The DupLEX-A<sup>TM</sup> system has the advantage of reduced false positives and the presence of an HA tag downstream of the B42 activation protein for subsequent purification.

**Example 6 - Transfection:** Using the pRC/CMV (Invitrogen) expression vector, COS-7 cells (CaPO4, glycerol shock and chloroquine; and lipofectamine), UC729.6 cells (electroporated using 280V and 960  $\mu$ F across 0.4 cm) and P388D1 (CaPO4, glycerol shock and chloroquine and lipofectamine) have been transiently, and transfected P3881D1 cells using both CaPO4, glycerol shock and chloroquine and lipofectamine have been stably transfected. For stable transfectants, the FACS Vantage is used for flow cytometry with receptor-specific mAbs to cell sort for subpopulations expressing quantitatively different levels of receptor. It is appreciated that comparable levels of transfectant expression are required in studying differences in function by different receptor constructs.

**Example 7- Receptor and Isoform cloning:** Products generated by PCR are separated by electrophoresis through low melting point agarose. To generate clones for sequencing the TA Topoisomerase Cloning system (Invitrogen) is used. For mammalian expression, blunt end ligated PCR product is inserted into vector pRC/CMV (Invitrogen), transformed competent E. coli with ampicillin based selection and confirmed plasmid integration with plasmid isolation and restriction fragment pattern analysis. Newer strategies for eukaryotic expression including the use of unidirectional TA cloning strategies and the expression vector pCR3-Uni which uses the immediate-early CMV promoter (Invitrogen) are also suitable.

**Example 8 - Construction of GST fusion proteins:** To generate glutathione-S-transferase (GST) fusion constructs for expression in E. coli, PCR amplified cDNA or cDNA fragments are subcloned into the BamHI-EcoRI site of the pGEX-2T vector (Pharmacia Biotech). Following transformation into E. coli

DH5a, fusion protein is induced in log phase cultures by the addition of 0.1 mM isopropyl-b-d-thiogalactopyranoside and purified by incubation of soluble lysates with glutathione-sepharose beads.

**Example 9 - Site-directed mutagenesis:** To generate site-specific mutations, oligonucleotide primers containing the desired mutation(s) are used for PCR of  $\alpha$ -chain cDNA. With the PCR Site-directed Mutagenesis System (Life Technologies), the amplified fragments are cloned in plasmid pAMP2 using Uracil DNA glycosylase and used to transform competent *E. coli*. Mutations are verified by sequencing and then cloned into an appropriate eukaryotic expression vector such as pRC/CMV to generate stable transfectants of P388D1 cells.

**Example 10 - Statistics:** Experimental data is evaluated statistically by means of StatView (Abacus Concepts, Inc., Berkeley, CA) or InStat (GraphPad Software, San Diego, CA) programs run on computers. Student's t tests for comparison of means of two data sets, ANOVA for multiple comparisons, and regression/correlation analyses will be performed as appropriate. Simple transformations (e.g. logarithmic) will be performed as needed to improve the distribution characteristics of the data ('normalizing'). Non-parametric methods are also available.

**Example 11 - Alternative Identification Method for Fc $\alpha$ RIa genotype:** Alternative methods of genotyping are also operative, such as the oligonucleotide ligation assay (OLA). In this genotyping assay, the polymorphic residue is within the PCR amplicon. Three oligonucleotides are then added; one that is immediately 3' to the polymorphic residue, a fluorescently labeled

oligonucleotides that is complementary to one allele at the 3' end and a fluorescently labeled third oligonucleotides that is complementary to the other allele at the 3' end. The two labeled primers need to be of different lengths (different by >2 nucleotides) and can be labeled with the same or different fluorescent probes (such as 6-fam and tet for detection on the ABI377). This technique is now widely used for analysis of mutations in the cystic fibrosis gene (63).

**Example 12 - Determination of cytokine promoter alleles:** While some microsatellites associated with various cytokine genes have been identified, a systematic search of the 5' promoter region reference sequences for novel SNPs is not available (4,45-51,113). However, SNPs occur with reasonable frequency (approximately 1 per 500-1000 bp) and that at least some of these SNPs are of biological significance. Identifying such SNPs and developing both microsatellite and SNP assays for characterization of a clinical population use the techniques of Examples 1-5, as well as other techniques conventional to the art. Examples of cytokine genes are given:

	<u>Gene</u>	<u>Length of 5'</u> <u>Promoter Reporter</u>	<u>Genbank</u> <u>Accession No.</u>	<u>Reference</u>
20	TNF $\alpha$	1,178 bp	L11698	133
	IL-1 $\alpha$	1,413 bp	X03833	134
	IL-1 $\beta$	4,991 bp	U26540	135
	IL-1ra	5,953 bp	X64532	136
	IL-6	1,222 bp	Y00081	137
	IL-10	4,181 bp	X78437	138,139

**Example 13 - General Protein Techniques:** Techniques for immunoprecipitation, SDS-PAGE, Western blotting, and related methods are

known to the art. MAb to Fc $\gamma$ R and Fc $\alpha$ R (CD89) are commercially available (Medarex, Biodesign International, Pharmingen). MAb and polyclonal Ab to src-family tyrosine kinases and syk are also available (UBI, Santa Cruz Biochemicals, Signal Transduction Labs).

5           **Example 14 -  $\gamma$ -chain co-immunoprecipitation:** Using detergent lysing conditions that have been shown to preserve the non-covalent interaction between  $\gamma$ -chain and Fc receptor (35,97), cells are lysed in buffer containing 1% digitonin (Wako) and protease inhibitors. CD89 is immunoprecipitated with anti-receptor mAb using standard techniques. Following SDS-PAGE separation and blotting,  
10            $\gamma$ -chain will be detected with a rabbit polyclonal anti- $\gamma$ -chain Ab that recognizes both human and murine  $\gamma$ -chain.

**Example 15 - Phosphotyrosine Analysis:** Stimulated or control neutrophils are lysed as previously described in Example 8 with the addition of the phosphatase inhibitor sodium orthovanadate (0.4 mM). Anti-phosphotyrosine or  
15           anti-target immunoprecipitates are separated by SDS-PAGE and transferred to nitrocellulose membranes essentially as described (126). Blots are detected with either anti-target Ab or anti-phosphotyrosine (mAb 4G10 or PY20) and detected with a HRP conjugated sheep-anti-mouse IgG followed by detection with an enhanced chemiluminescence reagent set (Amersham). In vitro kinase assays are  
20           performed after immunoprecipitation by washing the immune complexes in kinase buffer (20 mM MOPS, 5 mM MgCl<sub>2</sub>) and adding 10-15 $\mu$ Ci  $\gamma$ <sup>32</sup>P-ATP and unlabeled ATP. After a 1-15 min incubation, the immune complexes are spun, washed and separated by SDS-PAGE (140).

**Example 16 - Collagenase Assay:** The release of collagenase (MMP-1) from human neutrophils or monocytes is analyzed with the use of a standard sandwich ELISA (129), using plates coated with mAb COMY4A2. After incubating with samples of monocyte supernatant or standards, bound collagenase is detected by means of monoclonal antibody and HRP-conjugated polyclonal anti-collagenase antibody 647.

**Example 17 - Cytokine assays:** Levels of cytokines IL-1, IL-1ra, IL-6, and TNF $\alpha$  secreted in culture media are assayed by ELISA, using polyclonal (goat) and monoclonal anti-human cytokine reagents (R&D Systems, Minneapolis, MN). For murine cytokines secreted by transfected cells, anti-mouse cytokine reagents is obtained from Pharmingen (San Diego), or R&D Systems. The assays are calibrated against standards obtained from the same sources.

**Example 18 - Purification of Igs:** Human serum Igs are purified by chromatography using anion exchange (Mono Q), molecular sieve (Superose 6 or 12, or Sephacryl S-300), affinity (jacalin-HiTrap for IgA1; protein G-HiTrap for IgG), and immunoadsorbent (anti-IgM-HiTrap) columns in an FPLC apparatus (Pharmacia Biotech, Piscataway, NJ) or conventional columns. IgA subclasses are separated by means of a jacalin-HiTrap column which retains IgA1 while IgA2 passes; IgA1 is then recovered by elution with 0.1 M melibiose. Monomeric and polymeric IgA are separated by HPLC on a Biosep Sec-S3000 column (Phenomenex, Torrance, CA), or for larger quantities by FPLC on a Sephacryl S-300 Hi-Prep column. All these procedures are frequently used in this laboratory,



and are capable of achieving >99% purity of Ig isotypes (141,142). Purity and concentration are assessed by SDS-PAGE and ELISA (see above).

IgA myeloma proteins of both subclasses and in different molecular forms (monomers and a variety of polymeric configurations) are available from a collection kept by Dr. Jiri Mestecky. These are purified by essentially the same procedures as described for normal serum IgA.

**Example 19 - Assay of Ig and antibodies (ELISA):** To assay Igs, the plates are coated with anti-Ig antibodies of the desired specificity (Dako Corp., Carpinteria, CA), at optimal levels (1-10 ug/ml). The plates are blocked with 0.15% Tween 20 in PBS, and serially diluted samples, starting from a dilution appropriate to the sample and the expected analyte concentration, are applied overnight. Bound Ig is detected by means of peroxidase-conjugated antibody to the analyte, diluted to the previously determined optimal level in each case (usually 1:1,000-1:5,000), and applied for 4 h. When IgA subclasses are assayed, the bound Ig is detected with monoclonal anti-IgA1 or anti-IgA2 antibodies (Nordic Immunological Labs., Capistrano Beach, CA), followed by peroxidase-conjugated anti-mouse Ig (Southern Biotechnology, Inc., Birmingham, AL). Finally, the color developed with a substrate of o-phenylenediamine (0.5 mg/ml) plus 1 mM H<sub>2</sub>O<sub>2</sub> after 15 min is read at 490 nm in an ELISA plate reader (MRX; Dynatech Laboratories, Chantilly, VA) interfaced to a computer for data retrieval and processing. Controls include the use of uncoated (blocked) wells, and coated wells treated with all reagents but not exposed to analyte sample. All determinations are performed with at least duplicate samples. Where appropriate,

the assay is calibrated by means of a serially diluted standard (Human Ig Reference Preparation; The Binding Site, San Diego, CA), and a standard curve is generated by computer program based on the 4-parameter logistic method (143).

**Example 20 - Cell Preparations:** PMN, myeloid cells and monocytes  
5 are prepared as described (144) using discontinuous ficoll/hypaque density gradient centrifugation. Monocytes are purified by adherence to 100 mm tissue culture plates or directly in 96 well plates for use in functional assays for 90 min at 37°C/5% CO<sub>2</sub> (144). The non-adherent lymphocytes are recovered by rinsing in medium. When appropriate, adherent monocytes are collected by treatment for  
10 2 min. with 0.1% EDTA in Dulbecco PBS, and the cells washed and resuspended in medium. Alternatively, monocytes are allowed to form homotypic clumps by incubation 4°C (145). Clumped monocytes are then sedimented through a FCS cushion at 1 xg, washed and gently dispersed. Purities >90% can routinely be achieved with these techniques (144). The purity of monocytes is assessed by  
15 staining for non-specific esterase or with fluorochrome-labeled antibody to CD14. Cells are counted using 0.1% trypan blue to assess viability. For studies requiring large numbers of cells, buffy coat units from the blood bank are used which can yield in excess of 10<sup>8</sup> monocytes.

Eosinophils are isolated from the granulocyte fraction of citrated normal  
20 human blood (obtained by centrifugation through Percoll SG 1.082). Erythrocytes are removed by hypotonic lysis in water. CD16+ cells (mainly neutrophils, also NK cells) are removed by magnetic sorting (MACS column, Miltenyi Biotec)

using anti-CD16 antibody coupled to magnetic beads. The yield is ~98% pure eosinophils (146).

**Example 21 - Cell culture:** Cells are cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, non-essential amino acids, and antibiotics (complete medium) at 37°C in humidified 5% CO<sub>2</sub>/air (123,144).

**Example 22 - Flow Cytometry:** Standard analytical flow cytometry (one to four colors) is performed on a FACScan flow cytometer (Becton-Dickinson). Sorting of transfected cell lines is performed on a FACS-Vantage (Becton-Dickinson). The instrument is capable of routine sterile sorts into tubes or tissue culture plates.

**Example 23 - Phagocytosis Assay:** The phagocytic response of transfected murine cells or human peripheral blood monocytes are assessed by flow cytometry (41). Fluorescent 1 um microspheres (Polysciences, Warrington, PA) are coated with human IgA or IgG, or with mAb to CD89 at 25 ug/10<sup>9</sup> microspheres in PBS at 4°C for 16 h and blocked with 1% human serum albumin (HAS) in PBS. Control microspheres are coated with HSA only. Alternatively, microspheres are coated with anti-human light chain F(ab)'<sub>2</sub> to capture human Ig in a more physiological presentation of the Fc region for receptor engagement. The coated microspheres are added to the cultured monocytes at a ratio of 10 microspheres/monocytes for 4 h at 37°C in 5% CO<sub>2</sub>, and the cells are collected for analysis by flow cytometry (FACScan, Becton Dickinson). Alternatively, biotinylated anti-receptor mAb or Ig are bound to streptavidin saturated biotinylated PKH26-labeled E as we have previously reported (147). The

opsonized E are mixed with phagocytes at a ratio of 50:1, incubated for varying periods of time, and the non-internalized E are removed by hypotonic lysis. Phagocytosed E are then quantitated by flow cytometry.

**Example 24 - Superoxide Assay:** The production of superoxide ( $O_2^-$ ) by transfected cells of human monocytes is determined by superoxide dismutase (SOD)-inhibitable cytochrome C reduction (140). Cytochrome C (Sigma Chemical Corp., St. Louis, MO) is added to the monocyte culture medium, with or without SOD (Sigma), and the amount of reduced cytochrome C is measured by absorbance at 550 nm.

**Example 25 - Analysis of changes in intracellular  $Ca^{2+}$  levels:** Indo-1 (for cuvette based and flow cytometric  $[Ca^{2+}]$  determination) or Fura-2 (for single cell  $[Ca^{2+}]$  determination), both calcium binding fluorescent dyes whose spectral properties change with binding of free  $Ca^{2+}$ , is used to measure changes in intracellular calcium concentrations ( $[Ca^{2+}]_i$ ) (4,122,123,148). Cuvette based batch analysis is performed in an SLM-8000 spectrofluorometer and flow cytometric analysis is performed in the FACS-Vantage equipped with UV-excitation.

**Example 26 - Novel molecular associations with the cytoplasmic domain of  $Fc\alpha RI$ :** Using the yeast two-hybrid system based on the GAL4 transcriptional activator, the entire 41 amino acid cytoplasmic domain of CD89 in-frame with a 6-gly linker is cloned into the pGBT9 vector containing the DNA binding domain of the GAL4 transcriptional activator. Using this binding domain construct expressing the cytoplasmic domains of  $Fc\alpha RI$ , screening against cDNA

expression libraries that have been cloned into the pACT vector with the GAL4 activation domain (AD) occurs. Cloned cDNA libraries derived from total unstimulated human PBMC, EBV transformed pooled human PBMC and PHA stimulated human PBMC are utilized. Using strain HF7c yeast His auxotrophs, double transformants (BD-CD89 + AD-cDNA construct) are screened on Trp<sup>-</sup>, Leu<sup>-</sup>, His<sup>-</sup> dropout plates containing 10 mM 3-amino-triazole to select for positive interactions between the CD89 cytoplasmic domain and the fusion protein encoded by AD-cDNA. Growth positive colonies are then screened secondarily for LacZ, the second reporter under the control of the GAL4 promoter. The AD-cDNA construct plasmid is recovered from growth (+), LacZ (+) colonies by transforming into E. coli, harvested, sequenced, and compared to information currently in GenBank. Likely candidates, identified by multiple isolates and by putative biology, are directly rescreened (BD-CD89 + AD-candidate cDNA), and, if positive, are then tested for protein-protein interactions in co-immunoprecipitations from whole cell lysates and in GST-fusion protein affinity matrix/whole cell lysate interactions. Positive candidates identified in the two hybrid screen which are also positive for *bona fide* protein-protein interactions are deemed to be true associations. Subsequent studies depend on the structural and biological nature of the protein(s) identified, but such studies include truncations/point mutations to identify the regions in CD89 and in the interacting protein that mediate the interaction (in both the yeast two-hybrid system and/or transfection of mutant forms into mammalian cells), scanning laser confocal

microscopy to identify association in intact cells, and functional studies to demonstrate the biological importance of the interaction.

All positives, therefore, must be evaluated for direct protein-protein interactions in *in vivo* systems. Definition of these interactions requires suitable reagents. When mAb or polyclonal Abs directed against the interacting protein(s) are not immediately available, the initial characterization of the protein-Fc $\alpha$ RI interaction with a GST-fusion protein and polyclonal anti-GST Abs is carried out. Anti-CD89 monoclonal Abs are available, and rabbit polyclonal Abs directed against proteins identified in prior yeast two-hybrid screens are also readily made using conventional techniques.

The yeast two-hybrid system is best suited for higher affinity protein-protein interactions which are not phosphorylation dependent. Therefore, while true positives are highly informative, they do not comprise the entire repertoire of interacting proteins. The ability to identify true positives also depends on the suitability of the screening library. These libraries are particularly useful in identifying proteins that interact with Fc $\gamma$ R expressed in myeloid and lymphoid cells. When difficulties are encountered with the GAL4-based system, the DupLex-A<sup>™</sup> system has several potential advantages including a reduction in the number of false positives and ease of co-immunoprecipitation with the HA tag downstream of the B42 activation protein.

**Example 27 - Identification of novel SNPs in Fc $\alpha$ RI:** Through the examination of cDNA derived from disease free individuals, the present invention identifies multiple single nucleotide polymorphisms in the coding region of the

CD89 gene. Indeed, there are SNPs in each of the three exons encoding the EC1, EC2 and the TM/CY domains (Figure 4).

Amplification and sequencing of the CD89 cDNA is complicated by the presence of multiple alternatively spliced products. Currently available evidence suggests the presence of at least nine different alternatively spliced transcripts of Fc $\alpha$ RI (29,30,32,112,131). Many of these transcripts are co-expressed in the same cell type. Accordingly, the present invention amplifies the Fc $\alpha$ RI cDNA in two overlapping segments and gel purifies fragments corresponding to the full length Fc $\alpha$ RI transcript (expressing S1, S2, EC1, EC2 and TM/CY). The 5' segment is amplified with a pair of primers located in the S1 region and the EC2 region (nt 34 to 563). The 3' segment of the Fc $\alpha$ RI cDNA is amplified with a pair of primers located in EC1 and the 3'-untranslated region (nt458 to 934). The gel-purified PCR amplified segments are cycle-sequenced from both directions.

The sequencing of a specific PCR-amplified Fc $\alpha$ RI cDNA may be complicated by the presence of multiple splice variants that cannot be adequately resolved by agarose gel electrophoresis. For example, if there is a splice variant that does not express exon S2 (36 nt), which allows co-purification with wild type Fc $\alpha$ RI. Sequencing of the 5' segment in the forward direction is then not possible. However, in this situation, reverse direction sequencing up to the S2-EC1 splice site yields the necessary information.

**Example 28 - Expression of Fc $\alpha$ RIb and Fc $\alpha$ RI splice variants in patients with PD:** A protein product of a sequence is studied by biosynthetically labeling cells expressing the sequence with <sup>35</sup>S-methionine, the cells are lysed and

anti-CD89 mAb is used for immunoprecipitation from both cell supernatants and from cell lysates. Following deglycosylation with endoglycosidase F (N-Glycanase®), the putative product is resolvable from the larger  $\alpha$  isoforms on a higher percentage polyacrylamide gel.

5           Quantitative variations in the level of expression of these alternatively spliced forms of the Fc $\alpha$ RI mRNA are performed by RNase protection using a <sup>32</sup>P-labeled riboprobe that contains a portion of EC2 and a portion of the TM/CY domain. This strategy allows for resolution of the  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 and  $\beta$  forms by differences in electrophoretic mobility.

10           **Example 29 - Examination of Fc $\alpha$ RI post-translational processing in relation to IgA binding:** Fc $\alpha$ RI expressed on different cell types has distinct molecular weights which resolve to identical protein cores upon removal of N-linked oligosaccharides. For example, the mature proteins in <sup>125</sup>I-labeled anti-CD89 immunoprecipitates from PMN and eosinophils are each heterogeneous but  
15           very distinct in apparent Mr, 55-75kD vs. 70-100kD respectively (128). Upon treatment with endoglycosidase F, these forms reduce to a common major band of 32kD with a minor 36 kD band evident from PMN. The mol wt of Fc $\alpha$ RI from human monocytes and U937 cells are analyzed in comparison to PMN and eosinophils with and without endoglycosidase F (N-glycanase®) treatment as we  
20           have previously done for human Fc $\gamma$ RIII (144).

Two different independent approaches are used to analyze the quantitative aspects of IgA binding. First, the concentration dependence of human monomeric IgA, dimeric IgA, secretory IgA, and IgA1/IgA2 ligand in blocking the binding



of the anti-ligand binding site mAb My43 is determined. This approach has been used to define relative difference in affinity between the NK cell and macrophage glycoforms of Fc $\gamma$ RIIIa (CD16)(17). The alternative, more direct measure of binding affinity is performed with <sup>125</sup>I-labeled ligands and saturation binding isotherms. Using the technique of Weisbart et al. (149), the binding of 0-10 pmol of <sup>125</sup>I-labeled ligand to 5x10<sup>6</sup> cells for 24h at 4°C, in the presence and absence of excess unlabeled ligand, is measured. As an independent approach to determining of glycosylation alters binding of IgA, cells are treated with neuraminidase (150,151) and binding of IgA is analyzed (151).

**Example 30 - Modification of Fc $\alpha$ RI glycosylation in patients with PD and RA:** Fc $\alpha$ RI is immunoprecipitated with anti-receptor mAb and analyzed by SDS-PAGE and blotting with a variety of informative biotinylated lectins. PMN and monocyte Fc $\alpha$ RI are analyzed by lectin reactivity and comparisons are made between patients with PD, RA, IgA nephropathy and disease free individuals

The present invention demonstrates the CD89 polymorphism can be exploited as a diagnostic assay for determining susceptibility to and/or severity of various diseases involving IgA. The present invention further demonstrates a process through which the use of genotyping and/or phenotyping to assist in defining disease prognosis.

Any patents or publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent

as if each individual publication was specifically and individually indicated to be incorporated by references.

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One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objectives and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules and specific compounds described  
5 herein are presently representative of preferred embodiments, are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the appended claims.

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